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14. ABSTRACT

Studies on the association of Epstein-Barr virus with Breast Carcinoma have reported conflicting results. They have in fact raised questions about a geographic variance in this association and the technical challenges of localizing the virus in tissue. We have addressed both these questions in our study. To look for a geographic variance, we analyzed breast carcinoma cases from four countries: USA (n=100), India (n=100), China (n=79) and Saudi Arabia (n=23). Each case was subjected to Conventional and Real-Time PCR with EBNA-1. PCR positive cases were further analyzed by EBER in-situ hybridization to localize the virus and a few cases were studied by Laser Microdissection (LMD). Our overall PCR positivity rate was 35-55% and did not exhibit any significant geographic variance. EBER in-situ confirmed the presence of the virus in the tumor cells in only four cases. The remaining cases were harboring the virus in the lymphoid cells infiltrating the tumor. LMD showed the presence of EBV in EBER positive as well as some negative cases (all PCR positive cases) but EBV could also be demonstrated in the fibrous tissue. Considering the entire spectrum of results obtained in this study we conclude that EBV does not play any major role in the pathogenesis of breast carcinoma.

15. SUBJECT TERMS
Epstein-Barr virus (EBV), Breast cancer pathogenesis

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INTRODUCTION

Several studies have been done to explore a relationship between Breast Cancer and Epstein Barr Virus (EBV), however, two major issues still need to be addressed:

1) Geographic variability (as seen in other malignancies associated with EBV), and
2) Technical challenges in detecting EBV within the tumor cells.

We have evaluated both issues in our study. With most studies reporting a negative correlation between breast cancer and EBV (1-7), our aim was to look for geographical variations in this association. Therefore, we have studied samples from 4 countries, USA, China, India and Saudi Arabia. It has also been established that different assays used on the same sample yield different results in the detection of EBV (8). EBER in-situ hybridization, considered as the "gold standard" for EBV detection in other EBV related tumors, may not give definative results for EBV detection in breast cancer tumor cells. Several other commonly used methods, such as Southern blot hybridization and immunohistochemistry all have limitations. (12,13). Therefore, we have used a combined approach and subjected each sample to a routine PCR screening for EBNA-1, followed by a real-time quantitative PCR which allows measurement of the amount/copy number of viral DNA. PCR positive cases were then subjected to EBER in-situ hybridization (ISH) to localize the EBV and a few cases were then selected for laser microdissection (LMD). This was in accordance with a recent National Cancer Institute recommendation of combining real time quantitative PCR with Laser Microdissection to improve localization of viral DNA to benign or malignant components of a tissue sample (8).

BODY

We have analyzed a total of 302 cases from 4 different geographical areas, including 100 cases each from the USA and India, 79 cases from China and 23 cases from Saudi Arabia. All the samples were screened for the presence of EBV genome using primers flanking the EBV nuclear antigen 1 (EBNA-1) region. Real-time PCR was also performed using the same primer on the American, Chinese and Saudi Arabia cases. Real-time PCR was not carried out on the Indian cases (the last sample group to be studied) because our data did not show that real-time PCR has advantage on sensitivity compared with conventional PCR. PCR for the detection of the defective heterogeneous EBV was also performed on all these cohorts.
EBER in-situ hybridization was performed on all the PCR positive cases to determine whether the EBV expression occurs in the tumor compartment or in the infiltrating lymphocytes.

PCR RESULTS

We have an overall PCR positivity rate of 34-57% using the EBNA-1 primer.

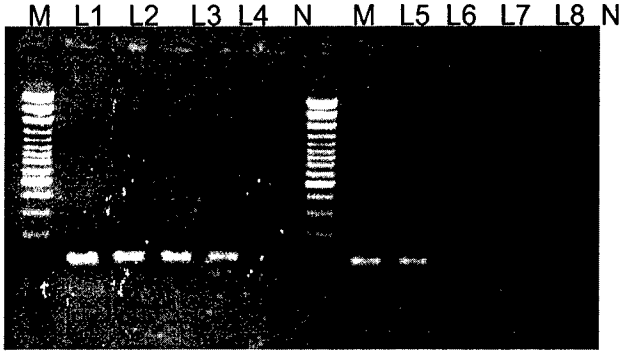
TABLE 1.

	CONVENTIONAL PCR	REAL-TIME PCR
AMERICAN CASES	45/100 (45%)	7/100 (7%)
CHINESE CASES	45/79 (57%)	39/79 (49.4%)
SAUDI ARABIA CASES	13/23 (56.5%)	5/23 (21.7%)
INDIAN CASES	34/100 (34%)	Not done

However, in a PCR reaction this positive signal could be generated by the presence of EBV in any component of the tissue. Therefore, we performed EBER in-situ hybridization on the PCR positive cases to localize the positive signal. It has been reported by earlier studies that this PCR positivity is more often from the lymphoid cells infiltrating the tumor than from the tumor cells themselves.

Also, as seen in Table 1, we had a discrepancy in results obtained by using the 2 different PCR assays, conventional and real time PCR, on the same samples. We investigated this further and came to the conclusion that the dUTP used in the dNTP mixture in the Taqman Universal Master Mix decreases the efficiency of the reaction. This is demonstrated in Figure 1. Here we set up 2 identical sets of PCR reactions. All the conditions and reagents were the same in both the reactions except for the dNTPs. The first set had the regular dNTP mixture (dATP, dGTP, dCTP, dTTP) while the second set had the dNTP mixture with dUTP in place of the dTTP. Both the sets were run in the same PCR machine at the same time so that all the PCR conditions were the same. 10 fold serial dilutions of the EBV positive Namalwa DNA were used as the positive controls.

FIGURE 1.



L1-L4 10 fold serial dilutions of Namalwa DNA (100ng, 10ng, 1ng, 0.1ng) with regular dNTPs

L5-L8 Same DNA dilutions but with dNTPs which have dUTP in place of dTTP.

N No template control
M 100 bp DNA marker

As seen in the above figure, the PCR product obtained using the dUTP (L5-L8) is significantly less as compared to that using the regular dNTPs (L1-L4). This finding suggests that conventional PCR is more suitable for our study where we are dealing with very low copy numbers of EBV in our samples. Therefore, we decided to perform only the conventional PCR on the Indian cases.

PCR performed for detection of the Heterogeneous EBV, showed a positivity rate of 1-13%. Therefore, we concluded that this form of EBV does not seem to play an important role in the development of Breast Carcinoma.

EBER IN-SITU HYBRIDISATION RESULTS

EBER in-situ hybridization was performed on all the PCR positive cases.

TABLE 2.

	AMERICAN CASES	CHINESE CASES	INDIAN CASES	SAUDI ARABIA CASES
EBER +VE [TOTAL NO. OF CASES]	12/45 (26.7%)	26/45 (57.8%)	13/34 (38.2%)	0/13
TUMOR CELLS +VE	4/12	0/26	0/13	-
LYMPHOID CELLS +VE	7/12	26/26	13/13	-

As can be seen in Table 2, the majority of the cases that were PCR positive were harboring the EBV in the lymphoid cells infiltrating the tumor, and not in the tumor cells. In our entire study we had only 4 cases (all from the American group) that showed the presence of the virus in the tumor cells. Of these 4 cases, only 1 case (US39) had 40-50% of the tumor cells positive for EBV (Figure 2). The remaining 3 cases had only occasional tumor cells positive by EBER. The Chinese, Indian and Saudi Arabian sample groups did not have any cases showing positive staining for EBER in the tumor cells. This finding reinforces previous reports indicating that EBV infection of the breast carcinoma cells is a very uncommon event. Even for positive cases, breast cancer is unlike "traditional" EBV-related tumors, which express EBER in virtually all tumor cells (8).

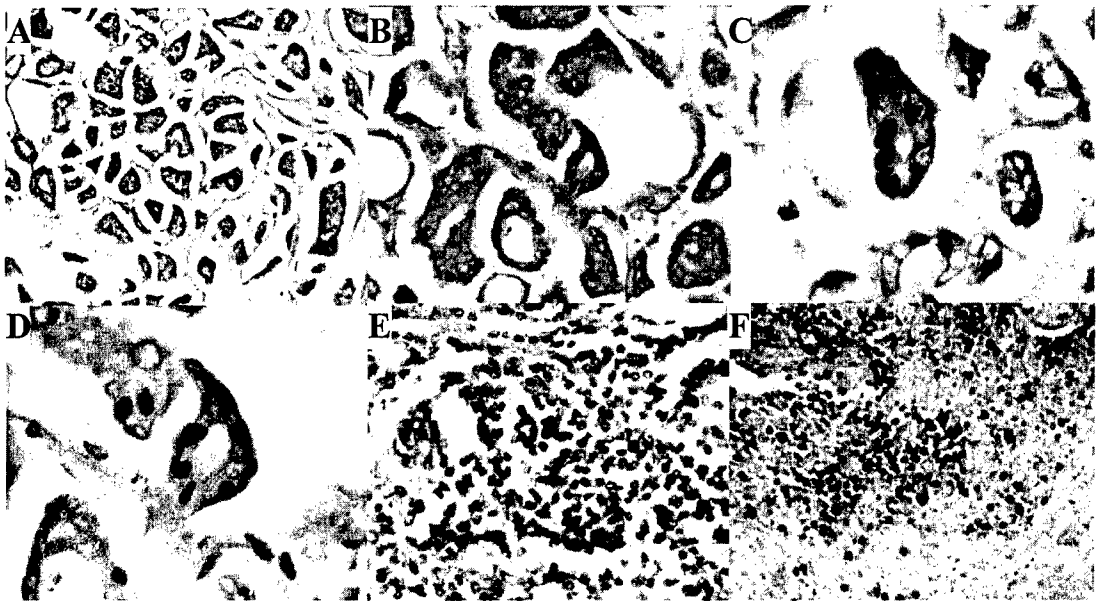


Figure 2. EBER-1 ISH in positive control and breast cancer sections. A, US case #39 shows nuclear staining of tumor cells (10X). B-D, same sections shown in higher power view. E shows positive staining of infiltrating lymphocytes in the tumor stroma, and F, positive control staining on a known EBV positive case.

However, we did make some incidental interesting findings while performing the in-situ hybridization studies. We demonstrated in rare cases, positive staining for EBER in endothelial cells, stromal cells and surrounding normal breast glands and in 1 case, in the normal skin epidermal cells over the breast tissue (Figure 3). This suggests that EBV can infect a broader spectrum of cells than previously recognized.

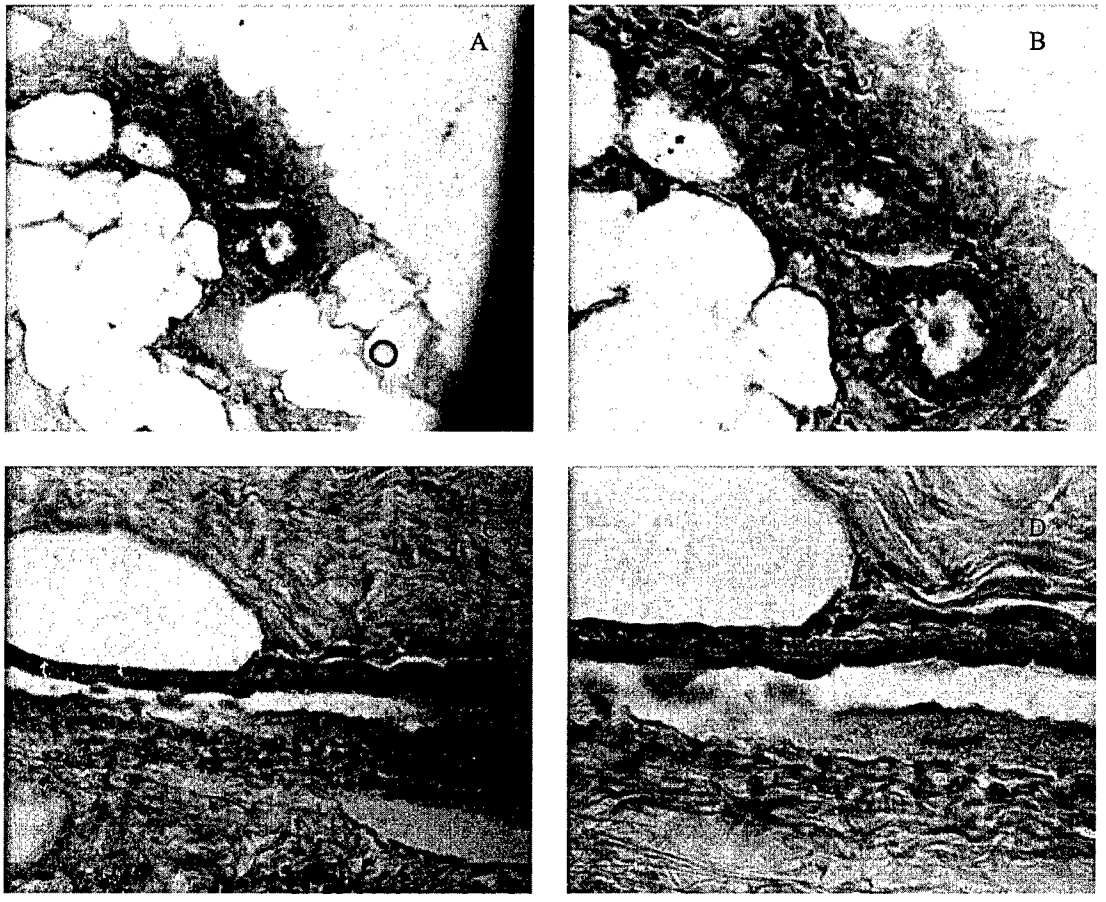


Figure 3. EBER ISH detected positive staining (dark blue cells) in some normal glandular cells in Panel A (low magnification) and Panel B (same section shown in higher magnification). In one case, some endothelial cells are positiv, Panel C (low power) and Panel D (high power).

LASER MICRODISSECTION

Previous studies suggest that breast tumors may express EBERs less abundantly than other malignancies (9,10) or have a previously unrecognized form of EBV infection characterized by EBER down regulation (2,11). Laser microdissection was performed on 2 groups of samples [as described in the Annual Report for 2004]. Samples that were PCR positive and EBER ISH positive in tumor cells and/or lymphocytes (group 1) vs PCR positive but EBER negative cases (group 2). PCR amplifying EBNA-1on the microdissected cells demonstrated high EBV positivity (2/5 in group 1 and 4/8 in group 2) in the tumor cells irrespective of the EBER ISH status.
Another interesting finding that we made on microdissection was that our acellular fibrous controls which were used as negative controls, also showed positive results on PCR with EBNA1 (Figure 2A and B). The " no template" negative controls run with the same reaction were always negative, ruling out contamination during PCR as a possible explanation.

FIGURE 4

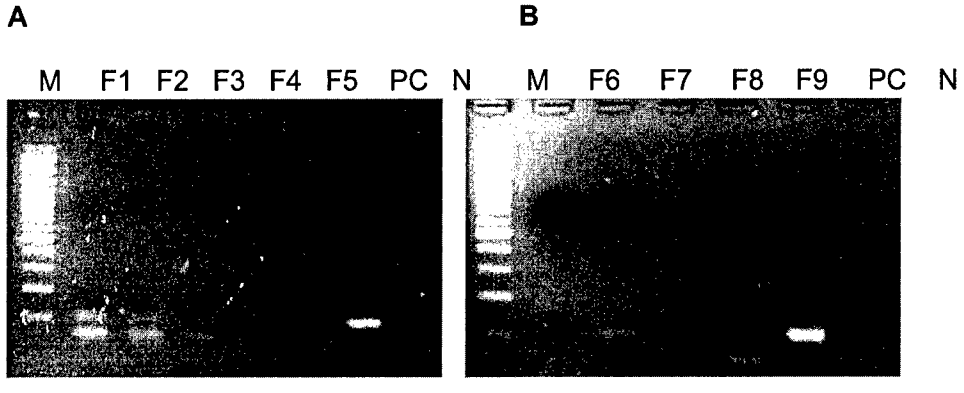


Fig 4. PCR for EBNA on microdissected fibrous tissue from 9 different samples (F1-F9).
PC: EBV positive Namalwa DNA used as a positive control
N: "No template" control
M: 100 bp DNA marker

As can be seen in figure4, 4 samples (F1, F2, F7, F9) showed positive bands for EBNA-1, while the "no template" control (N) run in the same reaction was negative.
In order to rule out the possibility of contamination during the laser microdissection procedure, we microdissected samples of the membrane (devoid of any tissue section) from the PEN membrane coated slides (Leica) used for microdissection. These were then subjected to a routine EBNA-1 PCR. There were no positive bands obtained.
These findings with the fibrous tissue suggest the possibility of "free virus" in the tissue and warrant further investigation. It also makes the interpretation of all microdissection studies difficult.

KEY RESEARCH ACCOMPLISHMENTS

- Established EBER in situ hybridization for localization of EBV infected cells, and found a wide spectrum of target cells for EBV.

- Studied cases from different geographical regions: 100 USA cases, 79 China cases, 23 Saudi Arabia cases and 40 of Indian cases of breast carcinoma for EBV by PCR and EBER ISH.

- Developed Real-Time PCR assay for the EBNA1 region and also an assay for the defective EBV.

- Integrated different assays (PCR, ISH, and microdissection) for the interpretation of data.

- Developed LMD techniques for the isolation of tumor cells and other cellular and non-cellular compartments in tissue sections.

- Concluded that EBV infection of breast cancer cells is rare and even in positive cases, it is a sporadic event. It is unlikely that EBV plays a significant role in the pathogenesis and progression of breast carcinoma.

REPORTBLE OUTCOMES:

An abstract titled " Epstein-Barr virus and breast cancer" was presented in the " Era of Hope – Department of Defense Breast Cancer Research Program meeting" on September 24-27, 2002 at Orlando, FL.

One technologist was trained for molecular biology and Laser microdissection techniques supported by this reward.

A manuscript is under preparation.

CONCLUSIONS

Our study showed an EBV positivity rate of around 35-55% by PCR on EBNA1 using whole tissue sections without significant geographic variance. PCR on microdissected tumor cells also detected virus in 40-50% of specimens of PCR positive samples irrespective of EBER status. However, the finding of microdissected acellular fibrous tissue giving positive results on EBV PCR raises a big concern about the specificity of findings on the tumor tissue. The data indicates that study on viral pathogenesis is more challenging than traditional methods suggested, especially with low viral load and multiple cell types that can harbor the virus. In conclusion, our data strongly argues against EBV playing a major role in breast cancer pathogenesis.

An interesting incident finding is that besides infiltrating lymphocytes, occasionally normal glandular cells and more often, endothelial cells were positive for EBV, suggesting that EBV may infect more cell types than previously suggested.

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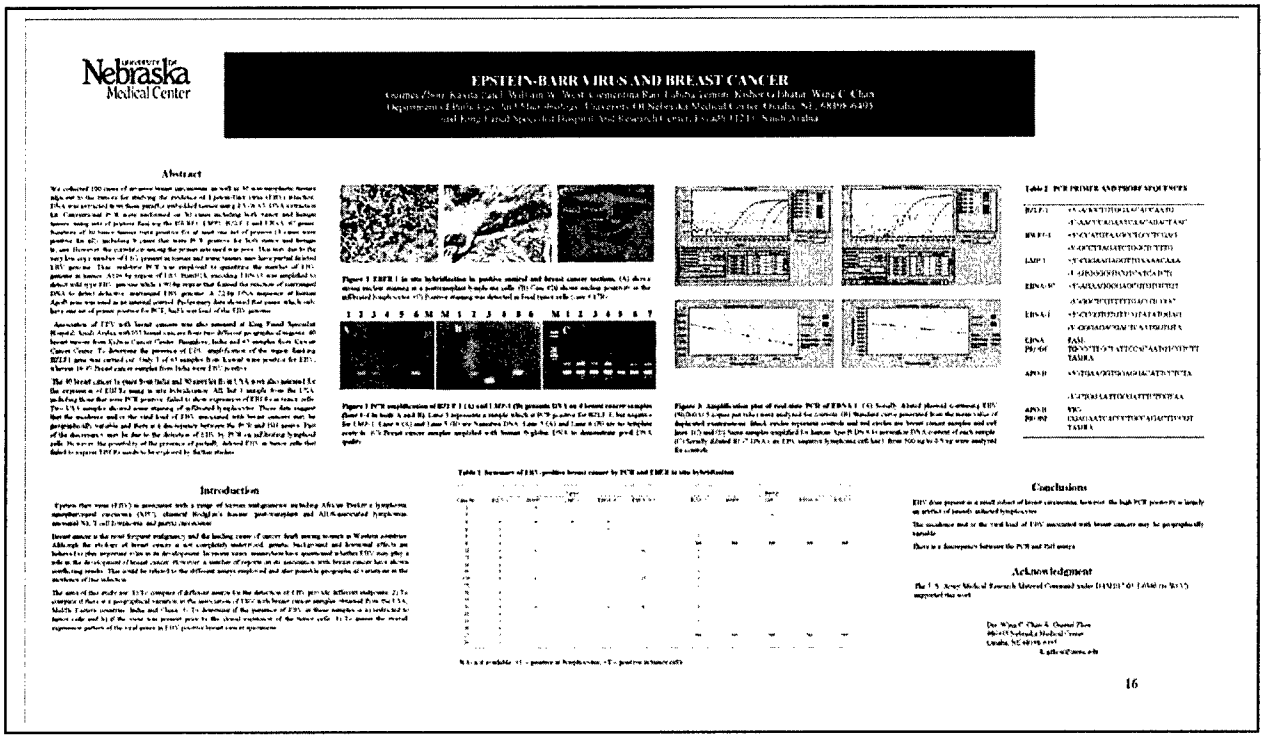
APPENDIX 1.

A poster titled " Epstein-Barr virus and breast cancer" was presented in the " Era of Hope – Department of Defense Breast Cancer Research Program meeting" on September 24-27, 2002 at Orlando, FL.



Nebraska Medical Center

EPSTEIN-BARR VIRUS AND BREAST CANCER

Abstract

Introduction

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Acknowledgment

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APPENDIX 2.

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